04-29-05 TRW 1647

Attorney's Docket No.: 13028-002001 / P12999

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Anke Rattenholl et al.	Art Unit	: 1647
Serial No.	: 09/807,096	Examiner	: Robert C. Hayes
Filed	: November 19, 2001		
Title	: METHOD FOR OBTAINING ACTIVE BETA-NGF		

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

TRANSMITTAL OF PRIORITY DOCUMENT UNDER 35 USC § 119

Responsive to Examiner's action of January 27, 2005, Applicants hereby confirm their claim of priority under 35 USC § 119 from the following application:

- European Application No. 98119077.0 filed October 9, 1998.

A certified copy of the application from which priority is claimed and a certified English translation thereof is submitted herewith.

Please apply any charges to deposit account 06-1050, referencing attorney docket 13028-002001.

Respectfully submitted,

Date: 4-28-05

Y. Rocky Tsao

Y. Rocky Tsao
Reg. No. 34,053

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110-2804
Telephone: (617) 542-5070
Facsimile: (617) 542-8906

21076899.doc

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EV411827853US

April 28, 2005
Date of Deposit

CERTIFICATE OF VERIFICATION OF TRANSLATION

I, DR. KERSTIN MAHLKE, OF SCHWABHAUSEN, GERMANY, AM THE TRANSLATOR OF THE EUROPEAN PATENT NUMBER EP 98 119 077.8, AND I STATE THAT THE FOLLOWING IS A TRUE TRANSLATION TO THE BEST OF MY KNOWLEDGE AND BELIEF.

.....

(Signature of Translator)

DATED THIS 18TH DAY OF APRIL, 2005.

BOEHRINGER MANNHEIM 4914/00/EP

P12999

A method for obtaining active ß-NGF

The present invention relates to a method for the preparation of ß-NGF by naturation of denatured inactive proNGF and cleavage of the prosequence.

Nerve growth factor (ß-NGF) is a neurotrophic factor required for the growth and survival of sympathetic and sensory neurons (Levi-Montalcini, R., Science 237, 1154 (1987); H. Thoenen and Y.-A. Barde, Physiol. Rev. 60, 1284 (1980); B. A. Yankner and E.M. Shooter, Annu. Rev. Biochem. 51, 845 (1982)). Furthermore, ß-NGF promotes the growth, differentiation and vitality of cholinergic neurons of the central nervous system (F.J. Hefti, J. Neurobiol. 25, 1418 (1994)). Possible therapeutic indications for recombinant human nerve growth factor include peripheral sensory neuropathies, e.g. associated with diabetes or as a possible side effect in AIDS therapy. Other indications for rh ß-NGF are central neuropathies, e.g. Alzheimer's disease. In this case, the loss of memory is the result of a loss of cholinergic neurons.

Human ß-NGF is translated as a preproprotein consisting of 241 amino acids. The prepeptide (18 amino acids) is cleaved off during translocation into the endoplasmic reticulum (ER), while the resulting proprotein is subsequently processed at its N an C termini (removal of the prosequence (103 amino acids) and the last two amino acids). Therefore, mature human NGF consists of 118 amino acids. It shows homology to murine ß-NGF and differs from this protein only by 12 amino acid substitutions. For conducting clinical studies or in a possible use as a therapeutic, homogenous ß-NGFs must be available in high amounts. A natural source of higher amounts of this factor are the submaxillary glands of male mice. These preparations, however, are heterogeneous mixtures of different dimers and therefore are unsuitable for therapeutic use. Furthermore, it is desirable to administer the human form of the protein to patients. In human tissue, however, neurotrophic factors are present only in minute concentrations.

Therefore, to use ß-NGF as a therapeutic agent recombinant preparation of the protein is the only possibility. This may be achieved in two ways: by a recombinant expression either in cell culture or in bacteria. Eukaryotic cell expression systems tend to provide only very low amounts

of proteins and are relatively expensive (J. Barnett, et al., J. Neurochem. 57, 1052 (1991); C. H. Schmelzer, et al., J. Neurochem. 59, 1675 (1992); R.H. Edwards and W.J. Rutter, United States patent No. 5,683,894).

In contrast, prokaryotic expression systems provide high amounts of the desired protein. However, in contrast to eukaryotic expression systems bacteria are unable to process the precursor proteins in the correct manner. As often in the expression of many other recombinant mammalian genes, the production of recombinant ß-NGFs in bacteria results in a biologically inactive translation product which is then accumulated in the cell in the form of aggregates (so-called "inclusion bodies" (IBs)).

Naturation of mature ß-NGF from such inclusion bodies, however, is only possible in the case of very low protein concentrations (below 10 µg/ml) and very low yields (up to about 10%). Such methods are for example described in EP-A 0 544 293, US patent 5,606,031, US patent 5,235,043, as well as WO 97/47735. The naturation via sulfitolysis of neurotrophic factors of the NGF/BDNF family is described in WO 95/30686.

In WO 97/47735 there is described an improved method for the naturation of proteins. In this method, the inactive protein is dissolved in a solution of a denaturing agent having a denaturing concentration in the presence of a low molecular weight substance which contains thiol groups. Afterwards, the dissolved protein is transferred from the strongly denaturing solution into a solution which is not or only weakly denaturing in which it assumes a biologically active conformation wherein the disulfide bonds are opened by means of the thiol component and are subsequently formed anew intramolecularly within the protein in such a manner that the protein assumes a conformation which has biological activity. Using an improved method of this type, a yield of naturation of ß-NGF of about 10% may be achieved.

It is an object of the present invention to provide an improved method for the preparation of ß-NGF which is simple and provides active NGF in a high yield.

This object has been achieved by providing a method for the preparation of a biologically active ß-NGF by means of naturation of the pro form present in an inactive and poorly soluble form wherein the pro form (proNGF) is preferably available in the form of inclusion bodies after

recombinant production in prokaryotes, said method being characterized by dissolving proNGF in its inactive, poorly soluble form by means of a solution of a denaturing agent having a denaturing concentration, transferring the proNGF into a solution which is not or weakly denaturing while maintaining the solubility wherein the dissolved denatured proNGF assumes a biologically active conformation which is determined by the disulfide bonds present in the native β-NGF, and afterwards cleaving off the prosequence whereby active NGF is obtained which may be isolated.

Surprisingly, it has been found that during naturation of inactive ß-NGF in vitro the prosequence has an essential and positive effect on the naturation process and that is possible according to the present invention to perform the renaturation in the most simple way and yet to achieve thereby yields of natured active ß-NGF which have not been known so far and have not been deemed possible.

The term "proNGF" means ß-NGF which is linked to its prosequence at its N terminus. According to the present invention, as the prosequence either the whole prosequence is used (R.H. Edwards and W.J. Rutter, US patent 5,683,894; A. Ullrich, et al., Nature 303, 821 (1983); SWISS-PROT protein sequence database No. P01138) or portions thereof, preferably complete domains. U. Suter et al. (EMBO J. 10, 2395 (1991)) have performed a more detailed study of the in vivo function of the pro peptide of murine ß-NGF with respect to the correct secretion in a COS-7 cell culture system. For this purpose, the prosequence was divided into five regions. Mutants were prepared in which one or more of these sequences were deleted. It has been found that the sequence regions containing amino acids -52 to -26 ("domain I") as well as -6 to -1 ("domain II") are essential for the expression and secretion of biologically active ß-NGF. Domain I is important for expression while domain II is required for correct proteolytic processing. It has been surprisingly found that proNGF has an activity in vivo in an analogous manner to ß-NGF. Therefore, proNGF may also be used as a therapeutic.

Inactive, poorly soluble proNGF is formed during overexpression of the protein in the cytosol of prokaryotes. In this case, proNGF prepared by recombination remains in the cytoplasm in an insoluble and aggregated form. These aggregates of proteins, the isolation thereof as well as their purification are described for example in F.A. Marston, Biochem. J. 240 (1986) 1-12. To

isolate these inactive protein aggregates (inclusion bodies) the prokaryotic cells are disrupted following fermentation.

Cell disruption may be performed by conventional methods, e.g. by means of sonication, high pressure dispersion or lysozyme (R. Rudolph, et al.: Folding proteins. In: T.E. Creighton, (ed.): Protein Function: A Practical Approach. Oxford University Press, pp. 57-99 (1997)). It is preferably carried out in a buffer solution suitable for adjusting a neutral to weakly acidic pH value and serving as a suspension medium, such as 0.1 mol/l Tris-HCl. After cell disruption, the insoluble components (inclusion bodies) are removed in any suitable manner, preferably by centrifugation or filtration following one or more washing steps with agents that leave IBs intact but dissolve foreign cellular proteins as much as possible, e.g. in water or phosphate buffer, optionally with mild detergents such as Brij® added. Afterwards, the insoluble fraction (pellet) is subjected to the method according to the present invention for solubilization and naturation.

As the denaturing agent there is conveniently used a denaturing agent usually employed in the solubilization of inclusion body proteins. Guanidinium hydrochloride and other guanidinium salts, such as the thiocyanate, as well as urea and its derivatives are preferably used. Moreover, mixtures of these denaturing agents may be used.

The concentration of the denaturing agent is dependent on the type of the denaturing agent and can be easily determined by those skilled in the art. The concentration of the denaturing agent (denaturing concentration) is sufficient if complete solubilization of the denatured, poorly soluble protein can be achieved. For guanidinium hydrochloride, these concentrations usually are in the range of 3-8 moles/l, preferably 5-7 moles/l. For urea, the concentrations usually are in the range of 6-10 moles/l. By a weekly denaturing solution it is intended to mean a solution which contains a denaturing agent in a concentration which enables the formation of the correct disulfide bonds in the protein and thereby the formation of the native tertiary structure of the protein. Preferably, strongly and weekly denaturing solutions differ in their concentrations by a factor of 100 or more.

Furthermore, for complete monomerization of the inclusion body proteins it is advantageous to also add during the solubilization a reduction agent such as dithiothreitol (DTT), dithioerythritol

(DTE) or 2-mercaptoethanol in a concentration of 10-400 mM and particularly preferred in a concentration of 20-100 mM.

Following solubilization a dialysis is performed, preferably against a solution which contains a denaturing agent in a denaturing concentration in order to remove the optionally used reduction agent. Conveniently, the solution against which dialysis is carried out contains the denaturing agent in the same concentration as present in the denaturing solution.

The subsequent naturation according to the method of the present invention is performed at a pH in the neutral to alkaline range, preferably between pH 7 and 10, particularly preferred in a pH range between 7.5 and 9.5. As the buffer solutions, any conventional buffer may be used. Preferably, buffers known to those skilled in the art such as Tris or phosphate buffers are used as buffers during renaturation. To transfer the denatured protein into renaturation buffer, the solubilized protein is either diluted into the renaturation buffer or dialyzed against renaturation buffer. Thereby, the denaturing agent is also diluted down to a concentration (weakly denaturing solution) that further denaturation of the protein no longer occurs. Already during initial reduction of the concentration of the denaturing agent a renaturation process may take place. The conditions for the transfer of the protein into the solution which is not or only weakly denaturing must be properly selected to ensure that the protein substantially remains in solution. Conveniently, this may be achieved by a slow continuous or a stepwise dilution. In this respect, it is preferred to dilute the denaturing agent in such a manner that an as complete as possible naturation of the protein occurs or to almost completely remove the denaturing agent, e.g. by dialysis.

Preferably, naturation is performed in the presence of low molecular weight auxiliary agents having a positive effect on the yield during naturation. Such auxiliary agents are for example described in R. Rudolph, et al., US patent 5,593,865. Particularly preferably used as the low molecular weight auxiliary agent during naturation is arginine, conveniently in a concentration of 0.2 to 1.5 M.

The naturation according to the method of the present invention is preferably carried out by adding a thiol component in its reduced and oxidized forms. Preferred thiol components include glutathione in reduced (GSH) and oxidized forms (GSSG), cysteamine and cystamine, cysteine

and cystine or 2-mercaptoethanol and 2-hydroxyethyl disulfide. By the addition of these thiol reagents in reduced and oxidized forms it is possible to achieve both the formation of disulfide bonds within the folding polypeptide chain during renaturation and the "reshuffling" of wrong disulfide bonds within or between the folding polypeptide chains (Rudolph et al., 1997, loc. cit.).

Conveniently, the method according to the present invention is performed during naturation at low temperatures (preferably at about 10°C). In the method according to the present invention the renaturation is performed for a period of time of 0.5-5 h, preferably of 1-2 h.

To prevent oxidation of the reducing agent by oxygen present in the air and to protect free SH groups it is convenient to add a chelating agent such as EDTA, preferably in an amount of 1-20 mM, particularly preferred at about 10 mM.

By the term "activity of ß-NGF" it is intended to mean the biological activity of ß-NGF. Biologically active ß-NGF exists in the form of a dimer. The activity may be determined according to the DRG assay (dorsal root ganglion assay), R. Levi-Montalcini, et al., Cancer Res. 14 (1954) 49, and S. Varon, et al., Meth. in Neurochemistry 3 (9172) 203. In this assay the stimulation and survival of sensory neurons from dissociated dorsal root ganglia of chick embryos is monitored by means of neurite formation.

The prosequence represents a domain separate from the mature protein. Between these two domains there is an exposed protease cleavage site. These cleavage sites can be specifically processed by suitable proteases. For example, trypsin cleaves after basic amino acids such as lysine or arginine. If the ratio of proNGF to trypsin is appropriately adjusted the correctly folded, mature protein will not be degraded by this protease. In contrast, denatured proteins as well as folding intermediates expose sequences which are susceptible to an attack by the protease. Proteases having a trypsin-like substrate specificity are preferred for the processing of proNGF. These proteases cleave the protein without digesting the active portion of the protein molecule. As the trypsin-like proteases, several serine proteases (e.g. trypsin itself or γ-NGF) are considered. Trypsin is preferably used. For limited proteolysis, the protein is employed in a ratio of 1:40 to 1:2500 (trypsin:proNGF ratio) by weight while a range of 1:40 to 1:250 is preferred. The proteolysis is carried out using an incubation time of 1 min - 24 h, preferably 1 - 60 min at a temperature of 0°C to 37°C, preferably 0°C to 20°C. As the buffers those buffers are used which

do not inhibit the activity of the protease. Phosphate and Tris buffers in a concentration range of 10-100 mM are preferred. The limited proteolysis is performed in the range of the optimal pH value of the protease; a medium having pH 7-8 is preferred. After completion of the incubation time the proteolysis is stopped either by addition of a specific inhibitor, preferably 1-5 mM PMSF (phenylmethylsulfonylfluoride) or soy bean trypsin inhibitor, preferably 1 mg per 0.1-5 mg trypsin, or by reduction of the pH value to 2-3 by the addition of acid, preferably HCl (R. Rudolph, et al. Folding proteins. In: T.E. Creighton (ed.): Protein Function: A Practical Approach. Oxford University Press, pp. 57-99 (1997); R.H. Edwards and W.J. Rutter, US patent 5,683,894).

The following Examples, publications and Figures further illustrate the present invention the scope of which is obvious from the present Claims. The processes described are meant to be exemplarily only describing the object of the present invention also after modifications.

Figure 1 shows the proNGF plasmid construct pET11a-proNGF for the expression of recombinant human proNGF.

Figure 2 shows a Coomassie stain of an SDS PAGE gel (15%) with crude extracts of E. coli strain BL21 (DE3) pET11a-proNGF/pUBS520 prior to and after induction, respectively, as well as of an IB preparation (SDS PAGE according to U.K. Laemmli, Nature 227, 680 (1970)). U: crude extract prior to induction, I: crude extract after four hours of induction, P: IB pellet, S: soluble supernatant).

Figure 2a shows the effect of the pH value on the folding of rh proNGF at 10°C in 100 mM Tris/HCl, 1 M L-arginine, 5 mM GSH, 1 mM GSSG, 5 mM EDTA. The protein concentration was 50 µg/ml, the folding time was 3 hours. The mean values of two measuring series are shown.

Figure 2b represents the effect of different concentrations of L-arginine on the folding of rh proNGF. Renaturation took place at a pH of 9.5, the other conditions were identical to those used in pH variation. The mean values of two measuring series are shown.

Figure 2c shows the effect of different GSH concentrations on the folding of rh proNGF. The concentration of GSSG was 1 mM, the L-arginine concentration was 1 M. The other parameters of renaturation were identical to those used in arginine variation. The mean values of two measuring series are shown.

Figure 2d shows the effect of different GSSG concentrations on the renaturation of rh proNGF. The concentration of GSH was 5 mM. The other folding parameters were identical to those used in GSH variation. The mean values of two measuring series are shown.

Figure 2e shows the effect of different amounts of GdmCl on the yield of native rh proNGF. The amounts of GSH and GSSG were 5 mM and 0.5 mM, respectively. The other renaturation conditions were identical to those used in GSSG variation. The mean values of two measuring series are shown.

Figure 2f shows the effect of different protein concentrations on the yield of folding of rh proNGF. In all samples, the concentration of GdmCl was 200 mM. All other folding parameters were identical to those used in GdmCl variation. A single measuring series is shown.

Figure 3 shows the elution profile of the purification of rh proNGF by means of cation exchange chromatography on Poros 20 HS (Perseptive Biosystems, column volume 1.7 ml).

Figure 4 shows an SDS PAGE gel (15%, silver stain according to M. V. Nesterenko, M. Tilley, and S.J. Upton, J. Biochem. Biophys. Methods 28, 239 (1994)) of the purification of rh proNGF on Poros 20 HS (1: renatured proNGF as loaded onto the column; 2: void; 3: fraction 4 (66 to 69 ml); 4: fraction 5 (69-72 ml); 5: fraction 6 (72-75 ml); 6: fraction 7 (75-78 ml); 7: fraction 8 (78-81 ml); 8: fraction 9 (81-84 ml); 9: fraction 10 (84-87 ml)).

Figure 5 shows the UV spectrum of rh proNGF.

Figure 6 shows an IEX-HPLC elution diagram of rh proNGF (column material: Poros 20 HS, 100 mm x 4.6 mm column, Perseptive Biosystems company).

Figure 7 shows an RP-HPLC elution diagram of rh proNGF at 220 nm (Poros 10 R1 column, 100 mm x 4.6 mm, Perseptive Biosystems).

Figure 8 shows an SDS gel (15%, Coomassie stain) of the limited proteolysis of rh proNGF with trypsin (M: 10 kDA marker, 1: rh proNGF standard; 2: rh ß-NGF standard; 3: weight ratio trypsin:rh proNGF = 1:40, 4: 1:100, 5: 1:250, 6: 1:500, 7: 1:1000, 8: 1:2000, 9: 1:2500, 10: control without trypsin, with STI).

SEQ ID NOs: 1 and 2 show oligonucleotides for the construction of pET11a-proNGF.

SEQ ID NO: 3 shows the nucleotide sequence of the cDNA of human proNGF as well as the amino acid sequence of the translation product.

SEQ ID NO: 4 shows the amino acid sequence of the translation product.

Example 1

Cloning of the proNGF cDNA into an Escherichia coli expression vector

For cloning of the proNGF construct the T7 expression system of Novagen was chosen (F.W. Studier and B.A. Moffatt, J. Mol. Biol. 189 (1986) 113). The DNA sequence encoding proNGF is under the control of the strong T7 transcription signal. As the host strain, E. coli BL21 (DE3) is used. The chromosome contains the gene for T7 RNA polymerase. Expression of this RNA polymerase and thus of rh proNGF is induced by IPTG (isopropyl-ß-D-thiogalactoside).

The cDNA for human proNGF was obtained by PCR amplification from vector pMGL-SIG-proNGF of Boehringer Mannheim (PL No. 1905). An NdeI restriction site at the 5' end of the DNA sequence coding for proNGF and a BamHI restriction site at the 3' end were introduced by means of mutagenesis primers. The PCR product was inserted into the NdeI/BamHI restriction site of the multiple cloning region of vector pET11a (Novagen) (Fig. 1).

The following primers were used in the PCR:

Forward primer "FwProNGF":

5' - CG GAA TTC CA|T ATG GAA CCA CAC TCA GAG AGC - 3' **(SEQ ID NO: 1)**

Met Glu Pro His Ser Glu Ser

Reverse primer "RevNGF":

5' - CC G|GA TCC TTA TCA TCT CAC AGC CTT TCT AGA - 3' **(SEQ ID NO: 2)**

stop stop Arg Val Ala Lys Arg Ser

After cloning into the vector, the nucleotide sequence was verified by means of DNA sequencing.

Example 2

a) Expression of human proNGF in E. coli

For culturing the recombinant bacterial strain an overnight culture was prepared. For this purpose, a suitable volume of LB medium was added with 100 µg/ml ampicillin and 50 µg/ml kanamycin.

LB medium (1 l): 10 g trypton
10 g yeast extract
5 g NaCl

The medium was inoculated with a single colony and agitated over night at 37°C.

The next morning, the desired volume of 2xYT medium containing 100 µg/ml ampicillin and 50 µg/ml kanamycin was inoculated with the overnight culture in a ratio of 1:100 (v/v). The culture was agitated at 37°C and 200-250 rpm until an OD_{600} of 0.5-0.8 was reached. Afterwards, the expression of proNGF was induced by 3 mM IPTG for 4 h at the same temperature.

Subsequently, the cells were harvested by centrifugation and either disrupted immediately or stored frozen at -70°C.

2xYT medium (1 l): 17 g trypton
10 g yeast extract
5 g NaCl.

b) Isolation of IBs

The recombinant protein is present Ii the bacterial cells in the form of aggregates. The preparation of these "inclusion bodies" was performed according to R. Rudolph, et al.: Folding Proteins. In: T.E. Creighton (ed.): Protein Function: A Practical Approach. pp. 57-99.

For each cell disruption 5 g of the cell pellet were resuspended in 25 ml of 100 mM Tris/HCl pH 7.0; 1 mM EDTA. Afterwards, 1.5 mg of lysozyme were added per g of wet cell mass, incubated for 30 min at 4°C, and subsequently the cells were disrupted using a Gaulin cell disruptor. Then, 3 mM $MgCl_2$ as well as 10 µg/ml DNase were added to the crude homogenate and incubated for 30 min at 25°C. After DNase digestion the insoluble cell components were solubilized by addition of 0.5 volumes of 60 mM EDTA, 6% Triton X-100, 1.5 M NaCl pH 7.0 followed by incubation for 30 minutes at 4°C. The IBs were collected by centrifugation for 10 min at 13,000 rpm. Afterwards, they were washed for another four times each with 100 ml of 100 mM Tris/HCl pH 7.0; 20 mM EDTA and stored at -20°C.

In this manner, about 4 g of IB pellet could be reproducibly obtained from 10 l of E. coli culture (about 44 g wet cell weight). The preparations always contained approx. 90-95% rh proNGF (Fig. 2).

Example 3

a) Solubilization of IBs

400 mg of IB pellet were suspended in 2 ml solubilization buffer (100 mM Tris/HCl pH 8.0; 6 M GdmCl; 100 mM DTT; 10 mM EDTA), incubated for 2 h at 25°C and centrifuged for 30 min

at 13,000 rpm in the cold room. Afterwards, the supernatant was removed and adjusted to pH 3-4 with 1 M HCl. The solubilized material was dialyzed three times each against 300 ml 6 M GdmCl pH 4.0; 10 mM EDTA, i.e. twice for 2 h each at 25°C and once over night in the cold room (12°C, 16-18 h). The protein concentration was then determined using the method of Bradford (M.M. Bradford, Anal. Biochem. 72, 248 (1976)). The concentration of rh proNGF was between 40 and 50 mg/ml.

b) Optimizing the renaturation of rh proNGF

To prepare biologically active rh proNGF from the solubilized materials prepared in Example 3a) these were diluted into different renaturation buffers. To determine the optimal folding conditions, the following parameters were varied in the order listed:

a) temperature and time,

b) pH value,

c) arginine concentration,

d) GSH/GSSG concentration,

e) GdmCl concentration,

f) protein concentration.

The results are presented in Tables 1-2 as well as in Figs. 2a-f. The amount of renatured rh proNGF in the folding samples was determined by RP-HPLC. For this purpose, 925 µl of each of the folding samples were removed at predetermined time points and treated with 75 µl of 32% HCl to stop the folding reaction. For RP-HPLC analytics a Poros 10 R1 HPLC column and the Beckman Gold HPLC system with 125NM solvent module, 168 detector, autosampler 507, and analysis software "Gold V 8.10" were used. The elution peaks obtained were fitted using the "Peakfit" program version 2.01. For a quantitative determination of the yields, a standard graph was constructed using purified native rh proNGF. Since the rh proNGF IBs were very pure, the total amount of protein employed for renaturation was equated with the amount of rh proNGF in the quantitative analysis. The measurement results shown are mean values of two measurements each.

Table 1

Optimization of temperature and time during rh proNGF folding. The protein concentration in each of the renaturation samples was 50 µg/ml. The folding buffer consisted of

100 mM Tris/HCl pH 9.5,
1 M L-arginine,
5 mM GSH,
1 mM GSSG,
5 mM EDTA.

The measurement series were performed several times and fitted using an exponential function. The mean values of two measurements are shown.

Temperature [°C]	**Overall yield [%]**	**Plateau reached after about**	**rate constant k [s^{-1}]**
4	25.8	3.3 h	2.569×10^{-4} s^{-1}
10	29.0	1.6 h	4.865×10^{-4} s^{-1}
15	22.4	1.1 h	6.399×10^{-4} s^{-1}
20	12.0	1.0 h	1.065×10^{-4} s^{-1}
25	11.4	0.8 h	1.935×10^{-4} s^{-1}

Table 2

This Table shows the effect of different concentrations of GSH/GSSG (GSH = reduced glutathione; GSSG = oxidized glutathione) on the folding of rh proNGF. The renaturation buffer used was

100 mM Tris/HCl pH 9.5
1 M L-arginine
5 mM EDTA

The folding time was 3 h at 10°C. In the Table, the individual folding samples are presented in the order of decreasing yield. The average yields of two measurement series are shown.

No. of sample	ratio GSH/GSSG [mM]	yield [%]
1	5/0.5	37.7
2	5/1	35.0
3	5/5	34.0
4	5/2.5	33.1
5	1/1	29.4
6	5/10	27.6
7	5/20	26.0
8	2.5/1	22.1
9	10/1	21.2
10	1/5	18.9
11	20/1	10.9
12	0/1	9.85
13	0/0	0
14	5/0	0

c) Renaturation of rh proNGF on a preparative scale

Rh proNGF was renatured by dilution in folding buffer (100 mM Tris/HCl pH 9.5; 1 M L-arginine; 5 mM GSH; 0.5 mM GSSG; 5 mM EDTA). The folding was preformed at a protein concentration of 50 µg/ml. The renaturation sample was incubated for 3 h at 10°C.

d) Purification by means of ion exchange chromatography

The renatured material was dialyzed against 10 l of 50 mM Na phosphate pH 7.0; 1 mM EDTA (IEX buffer A) and centrifuged for 30 min at 20,000 rpm. The supernatant was loaded onto a Poros 20 HS column (1.7 ml) and eluted using a linear salt gradient (IEX buffer B: 50 mM Na phosphate pH 7.0; 1 M NaCl; 1 mM EDTA). The protein eluted at 980 mM NaCl (Fig. 3). Non-native rh proNGF can only be removed from the column using denaturing conditions.

Example 4

Characterization of rh proNGF

a) Determining the concentration and the molecular weight using UV spectrophotometry

To determine the concentration of rh proNGF in the purified samples, an UV spectrum from 240 to 340 nm was measured of samples dialyzed against 50 mM Na-phosphate pH 7.0; 1 mM EDTA (Fig. 5; the spectrum was recorded using a Beckman DU 640 spectrophotometer). The rh proNGF concentration of the sample was determined from the absorption at 280 nm. The calculation was based on a theoretical molar extinction coefficient of 25,680 l/(mol x cm) (calculated according to S.C. Gill and P.H. von Hippel, Anal. Biochem. 182, 319 (1989)) and a molecular weight of 24,869 Da per monomer (calculated by means of the ExPASy program "pI/Mw" and corrected for three disulfide bonds). The values obtained by means of the spectrum were in close correlation to the concentrations determined by the Bradford method. The molecular weight determination was carried out using electrospray mass spectrometry. The theoretical mass of recombinant proNGF is 24,689 Da. Experimentally determined were 24,871 Da.

b) Analysis of the purity and determination of the molecular weight using SDS polyacrylamide gel electrophoresis

15% polyacrylamide gels were used. Each sample contained 1% (v/v) 2-mercaptoethanol. In the SDS gel, recombinant human proNGF shows a slightly higher apparent molecular weight than expected: approx. 30 kDa (instead of 24.8 kDa) (Fig. 2).

c) Analysis of the purity by means of IEX-HPLC

24 µg (50 µl of a sample containing 0.48 mg/ml rh proNGF) of protein were loaded onto a Poros 20 HS column (125 x 4 mm) equilibrated with 50 mM Na phosphate pH 7.0; 1 mM EDTA, and were eluted at a flow rate of 5 ml/min with a linear gradient of 0 to 100% B (B = 50 mM Na phosphate pH 7.0; 2 M NaCl; 1 mM EDTA) in 10 minutes (Fig. 6). The absorption at 280 nm

was used for detection (Gyncotek HPLC system with Chromeleon version 3.14analysis software).

d) Analysis of the purity using RP C4 HPLC

3.1 µg of rh proMGF (15 µl rh proNGF having a concentration of 0.21 mg/ml) were loaded onto a Poros 10 R1 column (100 mm x 4 mm; Perseptive Biosystems) equilibrated with 0.13% (v/v) TFA. The protein was eluted at a flow rate of 0.8 ml/min with a non-linear gradient within 33 min (0-4 min: 6% B; 4-9 min: 6-30% B; 9-24 min: 30-69% B; 24-25 min: 69-100% B; 25-27 min: 100% B; 27-30 min: 100% B). As the eluent B there were used 0.1% (v/v) TFA in 80% (v/v) acetonitrile. The absorption at 220 nm was used for detection (Beckman "Gold" HPLC system with analysis software "Gold V 8.10"). Native rh proNGF eluted in a single peak at a retention time of 14.28 min (Fig. 7).

e) Analysis of the N terminal sequence

For N terminal sequence analysis the solubilized IBs which had been roughly purified by means of RP HPLC were used. The N terminal sequence was determined using an Applied Biosystems 476A Protein Sequencer. The following amino acid sequence was obtained:

H_2N-Met-Glu-Pro-His-Ser-Glu-Ser-Asn-Val

f) Biological activity of the recombinant human proNGF

The physiological activity of rh proNGF was determined using the DRG assay (= dorsal root ganglion assay) (R. Levi-Montalcini, H. Meyer, and V. Hamburger, Cancer Res. 14, 49 (1954); S. Varon, J. Nomura, J.R. Perez-Polo, and E.M. Shooter, Meth. In Neurochemistry 3, 203 (1972)). In this assay the stimulation and survival of sensory neurons from dissociated dorsal root ganglia of 7-8 day old chick embryos is determined by means of neurite formation. The rh proNGF sample was adjusted to concentrations of 0.019 to 20.00 ng/ml using culture medium. Per test sample 15,000 neurons were employed. After incubation for 48 hours at 37°C the number of surviving cells was determined. A solution of rh ß-NGF of known concentration was used as the reference sample. The quantitative evaluation is based on the so-called EC50 value,

i.e. the concentration of NGF where half of the neurons survive. For rh proNGF an EC50 value of 0.369 ng/ml is obtained. In comparison, the EC50 value obtained for the rh ß-NGF standard is 0.106 ng/ml. Considering the different molecular weights of rh ß-NGF and rh proNGF, the biological activity of mature rh ß-NGF is about twice as high as that of rh proNGF.

Example 5

a) Preparation of biologically active mature rh ß-NGF by limited proteolysis of rh proNGF

Human proNGF contains an arginine residue as the last amino acid of the prosequence. Therefore, mature rh ß-NGF can be obtained from this precursor by limited proteolysis in vitro using proteases of suitable substrate specificity such as trypsin.

500 µl of purified rh proNGF were dialyzed against 50 mM Tris/HCl pH 8.0. Following dialysis, a protein concentration of 0.49 mg/ml was measured by running an UV spectrum. Per digestion sample, 20 µg of proNGF were employed. After proteolysis, 3 µg (corresponding to 6 µl) of this sample were analyzed by means of SDS PAGE. As the trypsin stock solutions 0.1 µg/ml or 0.01 µg/ml, respectively, were used. The concentration of soy bean trypsin inhibitor (STI) was 1 mg/ml. Both proteins were provided in the form of lyophilisates (manufacturer: Boehringer Mannheim and Sigma, respectively) and were dissolved in the above-mentioned buffer.

Different weight ratios of trypsin/rh proNGF were used in the limited proteolysis (see Table 3). After an incubation for thirty minutes on ice each reaction was stopped by 5 µg STI. For control purposes rh proNGF without added protease was also incubated on ice, followed by addition of STI.

Table 3

Ratio trypsin:rh proNGF	m (trypsin) [µg]	V (trypsin) [µl]	V (rh proNGF) [µl]	V (STI) [µl]
1:40	0.5	5 (0.1 µg/ml)	40	5
1:100	0.2	2 (0.1 µg/ml)	40	5
1:250	0.08	0.8 (0.1 µg/ml)	40	5
1:500	0.04	4 (0.01 µg/ml)	40	5
1:1000	0.02	2 (0.01 µg/ml)	40	5
1:2000	0.01	1 (0.01 µg/ml)	40	5
1:2500	0.008	0.8 (0.01 µg/ml)	40	5
Control	-	-	20	2.5

g) Analysis of the cleavage products by N terminal sequencing

The digestion samples with a weight ratio of trypsin:rh proNGF of a) 1:40; b) 1:100; and c) 1:250 were analysed in more detail by N terminal sequencing. The two upper protein bands on the SDS PAGE gel (Fig. 8) correspond to trypsin (24 kDa) and STI (20.1 kDA), respectively. A band at 13 kDa contained several species:

N terminus 1: Met^{-104}....;

N terminus 2: Val^{-35}....;

N terminus 3: Ser^{1}....(mature rh ß-NGF);

N terminus 4: Gly^{10}....;

These peptides were present in different amounts in the different samples.

Sample a): N terminus 2:N terminus 3:N terminus 4 = 4:5:2.

Sample b): N terminus 2:N terminus 3 = 1:1; N terminus 4 in trace amounts.

Sample c) was additionally analyzed by means of RP C3 HPLC (column: Nucleosil 500-5 C3-PPN; 125 mm x 4 mm). Two peaks were obtained: peak 1 (12.32 min): N terminus 1; peak 2 (14.88 min): N terminus 2 and N terminus 3 in a ratio of 2:3.

To obtain mature rh ß-NGF from rh proNGF on a preparative scale 1.3 mg of rh proNGF (in 50 mM Tris/HCl pH 8.0; concentration 0.46 mg/ml) were added with trypsin in a weight ratio of 1:250 (trypsin:rh proNGF). The sample was incubated for 30 min on ice. Afterwards, the protease was inactivated by a 40 fold excess by weight of soy bean trypsin inhibitor. The cleavage sample was dialyzed against 50 mM sodium phosphate pH 7.0; 1 mM EDTA and then applied to a cation exchange column (1.7 ml Poros 20 HS; Perseptive Biosystems). In a linear salt gradient of 0 to 2 M NaCl the cleavage product eluted in a single peak. The elution at a salt concentration of about 840 mM NaCl corresponded to that of mature rh ß-NGF in a control experiment. The yield of purified cleavage product was 17%.

The biological activity of the purified cleavage product was tested by means of the DRG assay. It corresponded to the activity of mature rh ß-NGF (Table 4).

Table 4

Species	**EC50 value [pg/ml]**
rh ß-NGF	110
rh ß-NGF prepared by limited proteolysis of rh proNGF	171

References

Barnett, J., et al., J. Neurochem. 57 (1991) 1052

Bradford, M.M., Anal. Biochem. 72 (1976) 248

EP-A 0 544 293

Hefti, F.J., J. Neurobiol. 25 (1994) 1418

Hill, S.C. and von Hippel, P., Anal. Biochem. 182 (1989) 319

Laemmli, U.K., Nature 227 (1970) 680

Levi-Montalcini, R., et al., Cancer Res. 14 (1954) 49

Levi-Montalcini, R., Science 237 (1987) 1154

Marston, F.A., Biochem. J. 240 (1986) 1-12

Nesterenko, M.V., et al., J. Biochem. Biophys. Methods 28 (1994) 239

Rudolph. R., et al. (1997): Folding Proteins. In: T.E. Creighton (ed.): Protein function: A Practical Approach, pp. 57-99

Schmelzer, C. H., et al., J. Neurochem. 59 (1992) 1675

Studier, F.W., et al., J. Mol. Biol. 189 (1986) 113

Suter, U., et al., EMBO J. 10 (1991) 2395

SWISS-PROT Protein Sequence Database No. P01138

Thoenen, H., et al., Physiol. Rev. 60 (1980) 1284

Ullrich, A., et al., Nature 303 (1983) 821

US patent 5,235,043

US patent 5,593,856

US patent 5,606,031

US patent 5,683,894

Varon, S., et al., Meth. in Neurochemistry 3 (1972) 203

WO 97/47735

Yankner, B. A., et al., Annu. Rev. Biochem. 51 (1982) 845

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: BOEHRINGER MANNHEIM GMBH
(B) STREET : Sandhofer Str. 116
(C) TOWN:Mannheim
(E) COUNTRY: Germany
(F) POSTAL CODE : D-68305
(G) TELEPHONE:08856/60-3446
(H) TELEFAX: 08856/60-3451

(ii) NAME OF THE INVENTION: Method for the preparation of active beta-NGF

(iii) NUMBER OF SEQUENCES : 4

(iv) COMPUTER-READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM:PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30B (EPA)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : other nucleic acid
(A) DESCRIPTION : /desc = "Forward Primer - FwProNGF"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGGAATTCCA TATGGAACCA CACTCAGAGA GC 32

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : other nucleic acid
(A) DESCRIPTION : /desc = "Reverse Primer - RevNGF"

(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCGGATCCTT ATCATCTCAC AGCCTTTCTA GA 32

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 672base pairs
(B) TYPE nucleotide
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE : cDNA

(ix) CHARACTERISTIC:
(A) NAME/KEY : : CDS
(B) POSITION: 1..672

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
ATG GAA CCA CAC TCA GAG AGC AAT GTC CCT GCA GGA CAC ACC ATC CCC        48
Met Glu Pro His Ser Glu Ser Asn Val Pro Ala Gly His Thr Ile Pro
 1               5                  10                  15

CAA GTC CAC TGG ACT AAA CTT CAG CAT TCC CTT GAC ACT GCC CTT CGC        96
Gln Val His Trp Thr Lys Leu Gln His Ser Leu Asp Thr Ala Leu Arg
            20                  25                  30

AGA GCC CGC AGC GCC CCG GCA GCG GCG ATA GCT GCA CGC GTG GCG GGG       144
Arg Ala Arg Ser Ala Pro Ala Ala Ala Ile Ala Ala Arg Val Ala Gly
        35                  40                  45

CAG ACC CGC AAC ATT ACT GTG GAC CCC AGG CTG TTT AAA AAG CGG CGA       192
Gln Thr Arg Asn Ile Thr Val Asp Pro Arg Leu Phe Lys Lys Arg Arg
    50                  55                  60

CTC CGT TCA CCC CGT GTG CTG TTT AGC ACC CAG CCT CCC CGT GAA GCT       240
Leu Arg Ser Pro Arg Val Leu Phe Ser Thr Gln Pro Pro Arg Glu Ala
65                  70                  75                  80

GCA GAC ACT CAG GAT CTG GAC TTC GAG GTC GGT GGT GCT GCC CCC TTC       288
Ala Asp Thr Gln Asp Leu Asp Phe Glu Val Gly Gly Ala Ala Pro Phe
                85                  90                  95

AAC AGG ACT CAC AGG AGC AAG CGC TCA TCA TCC CAT CCC ATC TTC CAC       336
Asn Arg Thr His Arg Ser Lys Arg Ser Ser Ser His Pro Ile Phe His
            100                 105                 110

AGG GGC GAA TTC TCG GTG TGT GAC AGT GTC AGC GTG TGG GTT GGG GAT       384
Arg Gly Glu Phe Ser Val Cys Asp Ser Val Ser Val Trp Val Gly Asp
        115                 120                 125
```

```
AAG ACC ACC GCC ACA GAT ATC AAG GGC AAG GAG GTG ATG GTG TTG GGA      432
Lys Thr Thr Ala Thr Asp Ile Lys Gly Lys Glu Val Met Val Leu Gly
    130                 135                 140

GAG GTG AAC ATT AAC AAC AGT GTA TTC AAA CAG TAC TTT TTT GAG ACC      480
Glu Val Asn Ile Asn Asn Ser Val Phe Lys Gln Tyr Phe Phe Glu Thr
145                 150                 155                 160

AAG TGC CGG GAC CCA AAT CCC GTC GAC AGC GGG TGC CGG GGC ATT GAC      528
Lys Cys Arg Asp Pro Asn Pro Val Asp Ser Gly Cys Arg Gly Ile Asp
                165                 170                 175

TCA AAG CAC TGG AAC TCA TAT TGT ACC ACG ACT CAC ACC TTT GTC AAG      576
Ser Lys His Trp Asn Ser Tyr Cys Thr Thr Thr His Thr Phe Val Lys
            180                 185                 190

GCG CTG ACC ATG GAT GGC AAG CAG GCT GCC TGG CGG TTT ATC CGG ATA      624
Ala Leu Thr Met Asp Gly Lys Gln Ala Ala Trp Arg Phe Ile Arg Ile
        195                 200                 205

GAT ACG GCC TGT GTG TGT GTG CTC TCT AGA AAG GCT GTG AGA TGA TAA      672
Asp Thr Ala Cys Val Cys Val Leu Ser Arg Lys Ala Val Arg  *   *
    210                 215                 220
```

(2) INFORMATION FOR SEQ NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 224 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE: Protein

(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Glu Pro His Ser Glu Ser Asn Val Pro Ala Gly His Thr Ile Pro
 1               5                  10                  15

Gln Val His Trp Thr Lys Leu Gln His Ser Leu Asp Thr Ala Leu Arg
            20                  25                  30

Arg Ala Arg Ser Ala Pro Ala Ala Ala Ile Ala Ala Arg Val Ala Gly
        35                  40                  45

Gln Thr Arg Asn Ile Thr Val Asp Pro Arg Leu Phe Lys Lys Arg Arg
    50                  55                  60

Leu Arg Ser Pro Arg Val Leu Phe Ser Thr Gln Pro Pro Arg Glu Ala
65                  70                  75                  80

Ala Asp Thr Gln Asp Leu Asp Phe Glu Val Gly Gly Ala Ala Pro Phe
                85                  90                  95
```

```
Asn Arg Thr His Arg Ser Lys Arg Ser Ser Ser His Pro Ile Phe His
            100                 105                 110

Arg Gly Glu Phe Ser Val Cys Asp Ser Val Ser Val Trp Val Gly Asp
        115                 120                 125

Lys Thr Thr Ala Thr Asp Ile Lys Gly Lys Glu Val Met Val Leu Gly
    130                 135                 140

Glu Val Asn Ile Asn Asn Ser Val Phe Lys Gln Tyr Phe Phe Glu Thr
145                 150                 155                 160

Lys Cys Arg Asp Pro Asn Pro Val Asp Ser Gly Cys Arg Gly Ile Asp
                165                 170                 175

Ser Lys His Trp Asn Ser Tyr Cys Thr Thr Thr His Thr Phe Val Lys
            180                 185                 190

Ala Leu Thr Met Asp Gly Lys Gln Ala Ala Trp Arg Phe Ile Arg Ile
        195                 200                 205

Asp Thr Ala Cys Val Cys Val Leu Ser Arg Lys Ala Val Arg  *    *
    210                 215                 220
```

CLAIMS

1. A method for the preparation of a biologically active ß-NGF from its inactive, poorly soluble pro form which is obtainable after recombinant preparation in prokaryotes characterized in that proNGF in its inactive, poorly soluble form is dissolved by a solution of a denaturing agent in a denaturing concentration and is afterwards transferred into a solution which is not or weakly denaturing while solubility is maintained and wherein denatured proNGF assumes a biologically active conformation as determined by the disulfide bonds present in native ß-NGF, and subsequently the prosequence is cleaved off whereby active ß-NGF is obtained which can be isolated.

2. The method according to claim 1 characterized in that the not or weakly denaturing solution contains arginine.

3. The method according to claim 2 characterized in that the concentration of arginine is 0.2 to 1.5 mol/l.

4. The method according to claims 1 to 3 characterized in that the naturation is carried out in the presence of a thiol component in its reduced and oxidized form.

5. The method according to claims 1 to 4 characterized in that the cleaving off of the prosequence occurs by means of a protease having a substrate specificity for cleaving after the amino acid arginine.

6. The method according to claim 5 characterized in that trypsin is used as the protease.

7. The method according to claims 1 to 6 characterized in that guanidinium hydrochloride or urea is used as the denaturing agent.

Summary

A method for the preparation of biologically active ß-NGF starting with the proform proNGF is described. Following the expression of the proform of ß-NGF in a prokaryotic host cell the recombinant protein is isolated in the form of insoluble, inactive aggregates (inclusion bodies). After the solubilization thereof in a strong denaturing agent followed by conversion into the native conformation which is determined by the disulfide bonds present in the native ß-NGF and by subsequent cleavage of the prosequence biologically active ß-NGF is obtained.

Fig. 1

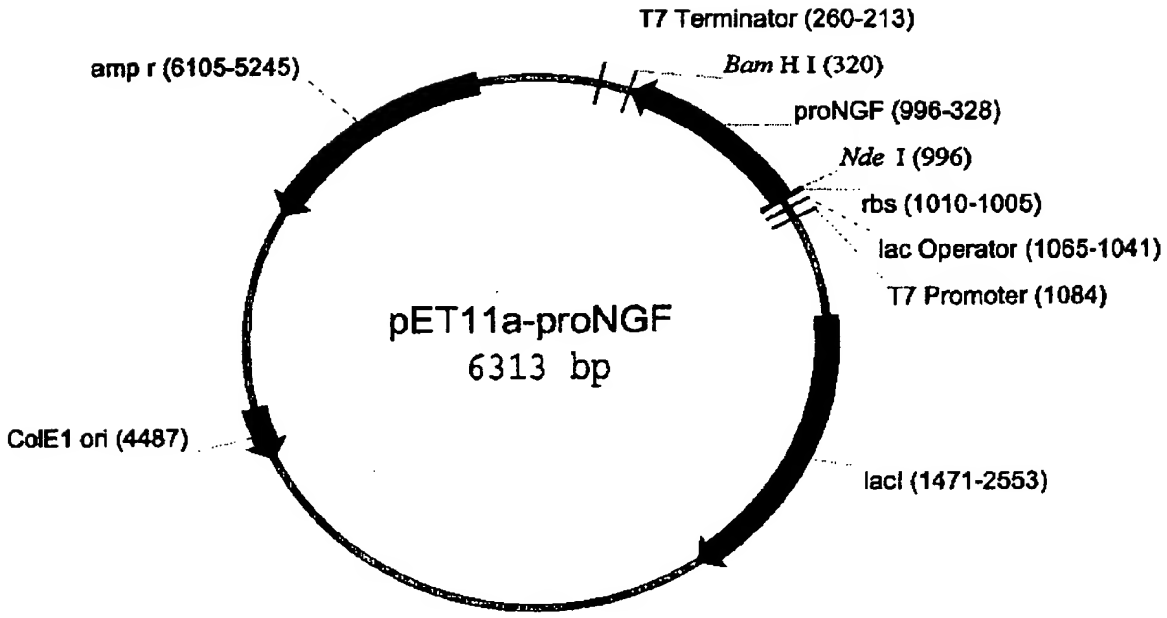

Fig. 2

Fig. 2a

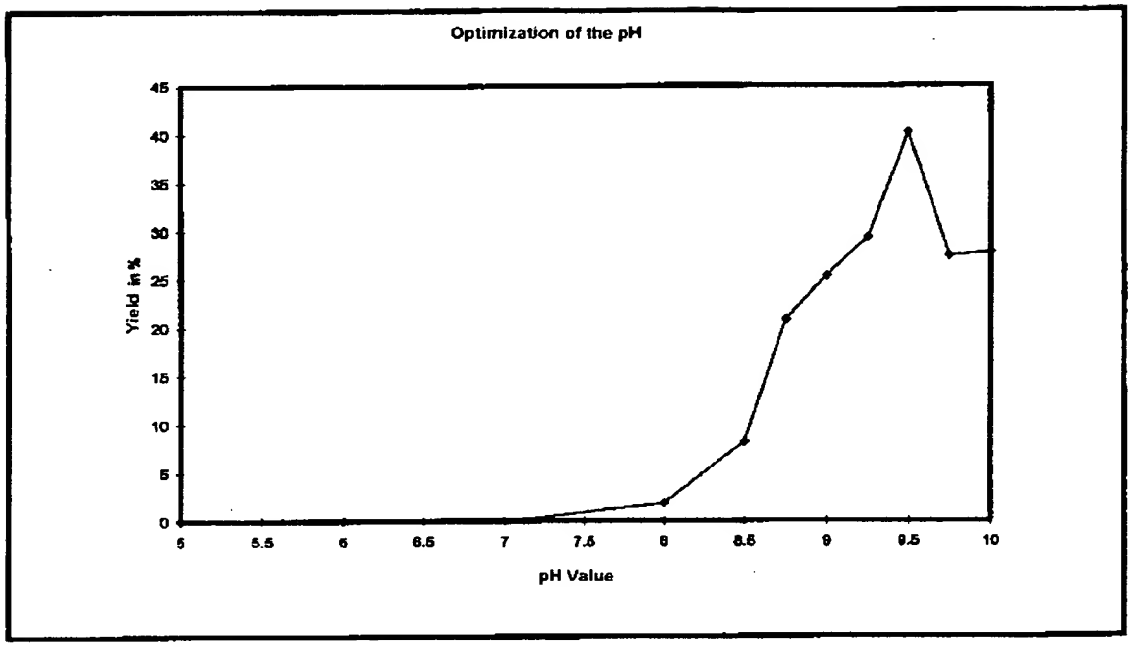

Fig. 2b

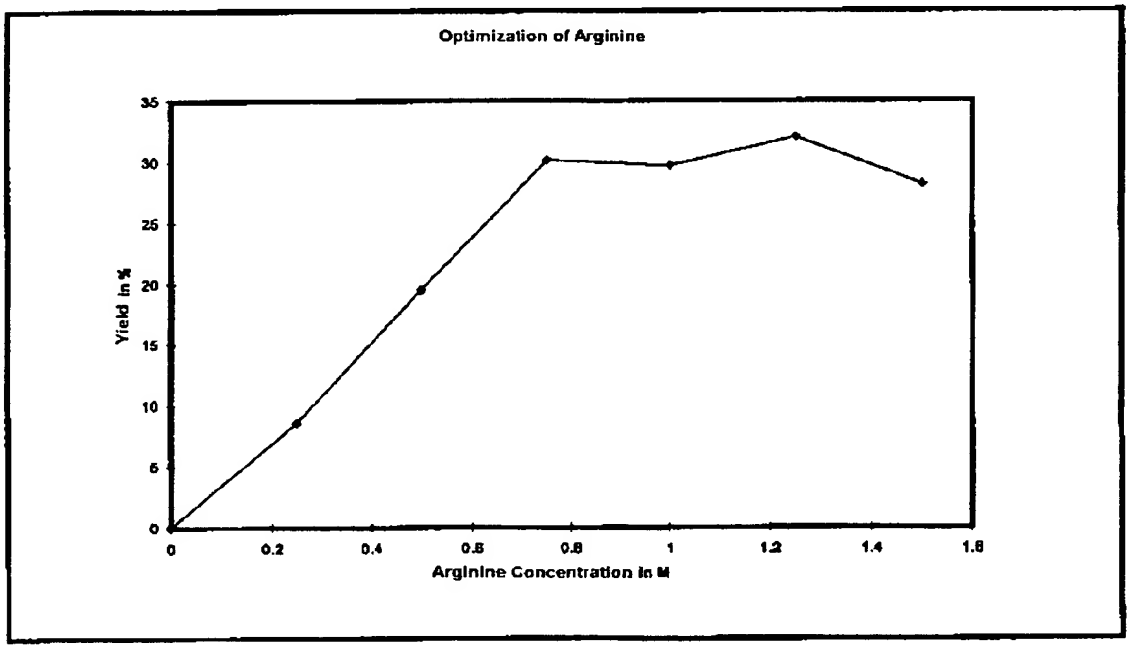

Fig. 2c

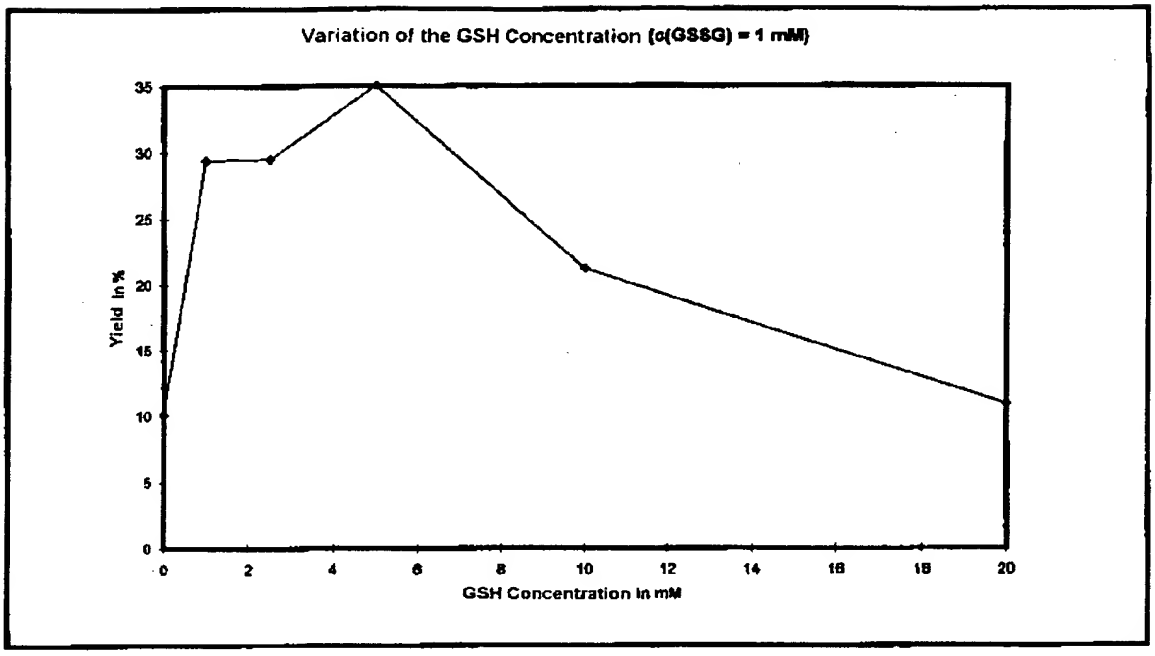

Fig. 2d

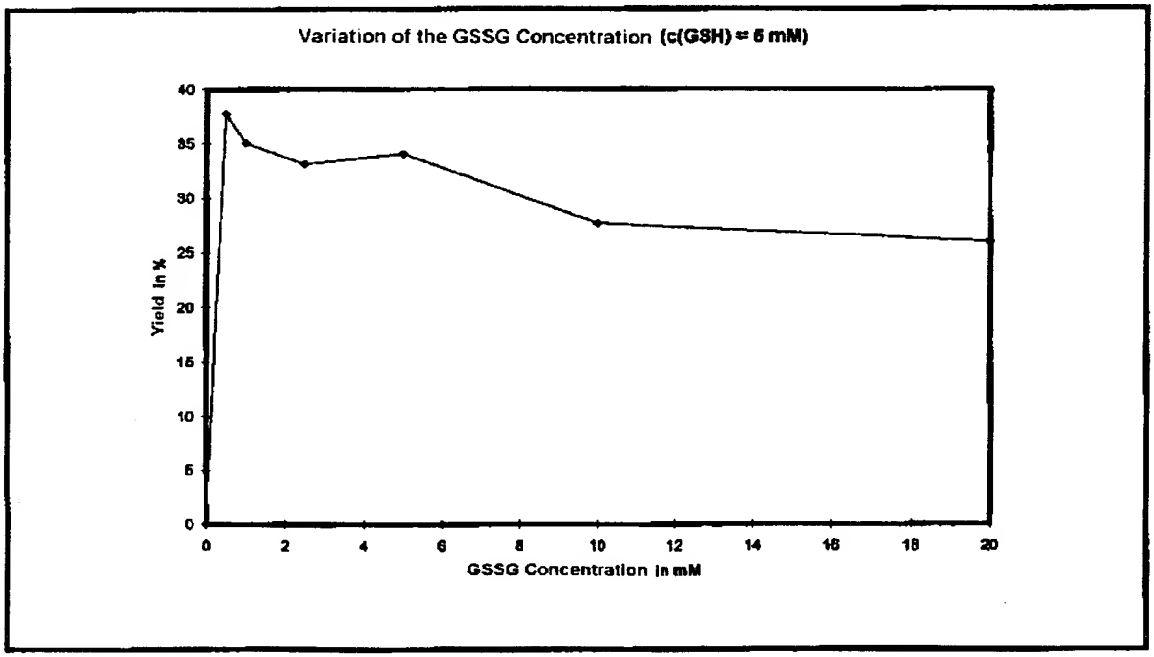

Fig. 2e

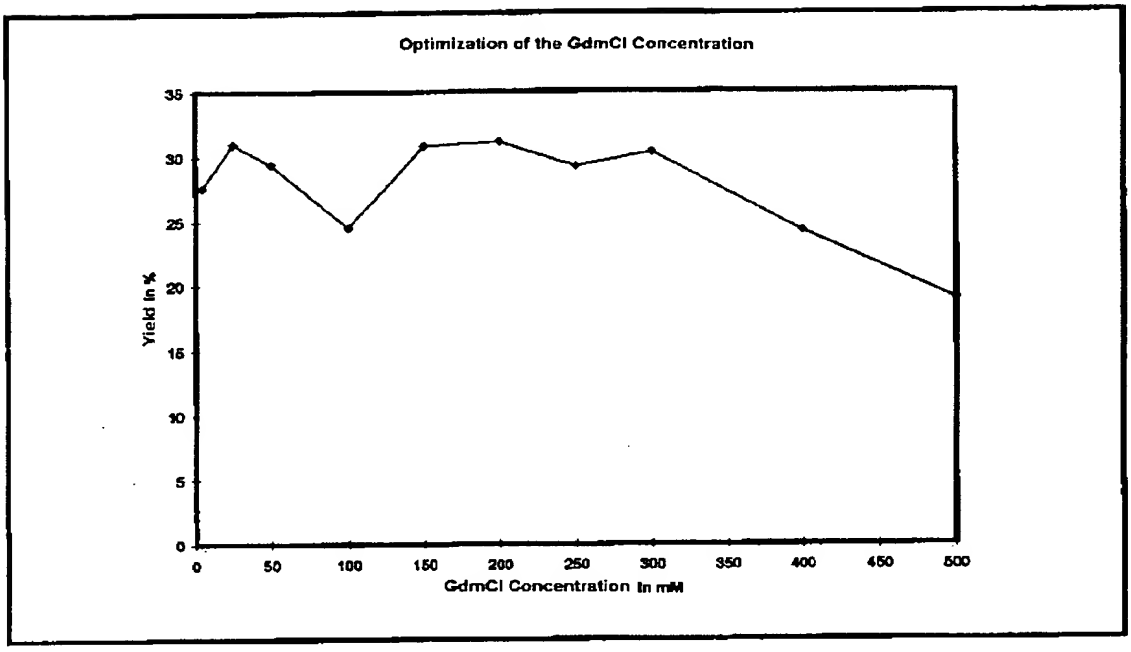

Fig. 2f

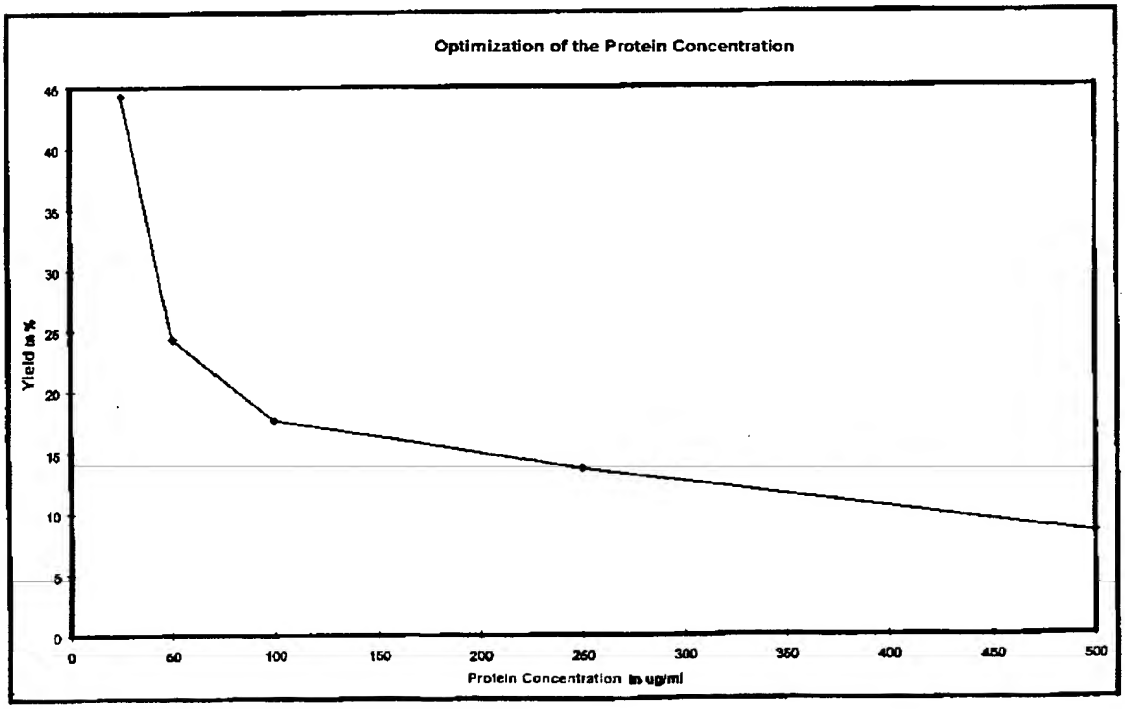

Fig. 3

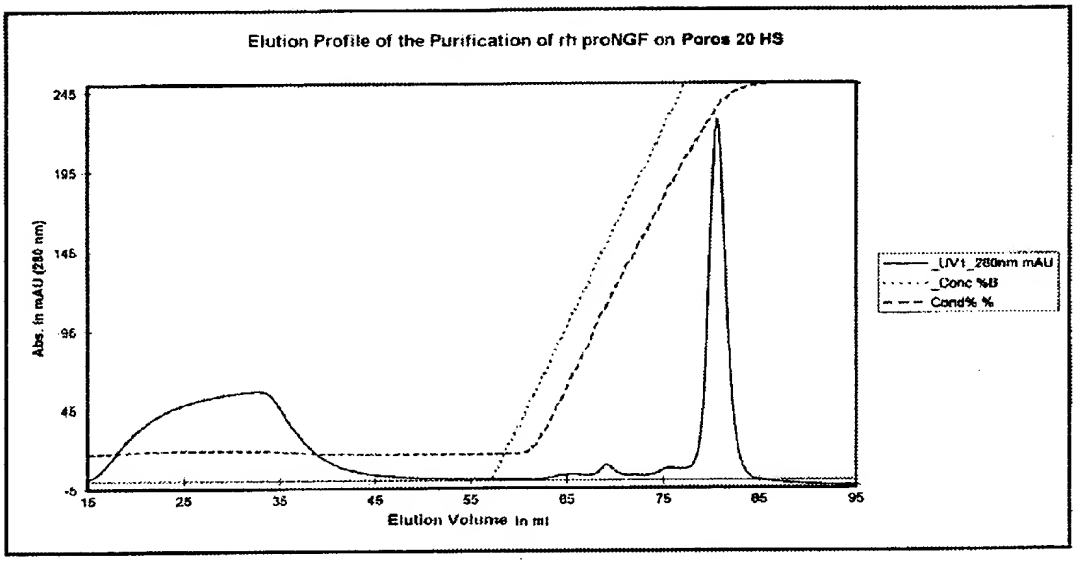

Fig. 4

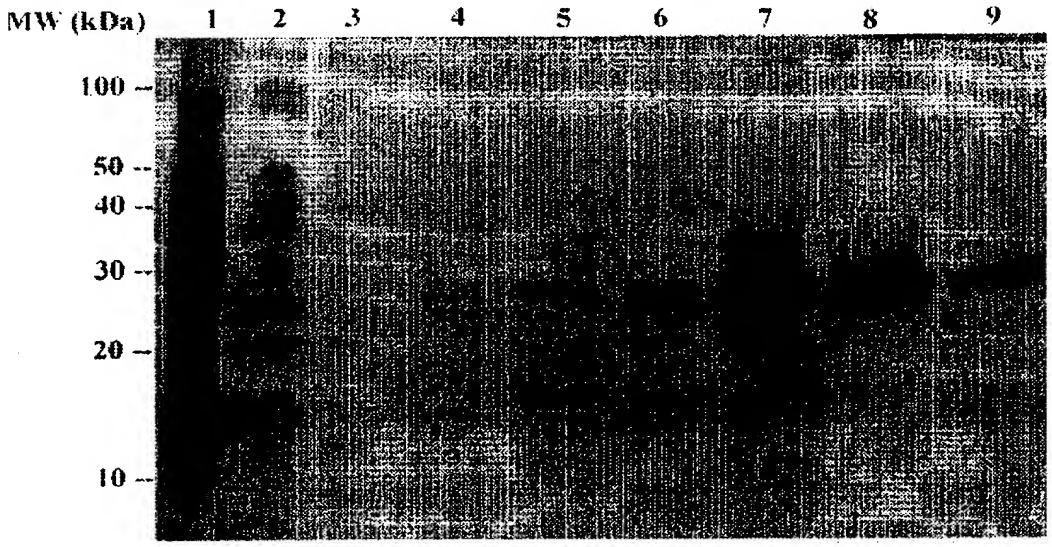

Fig. 5

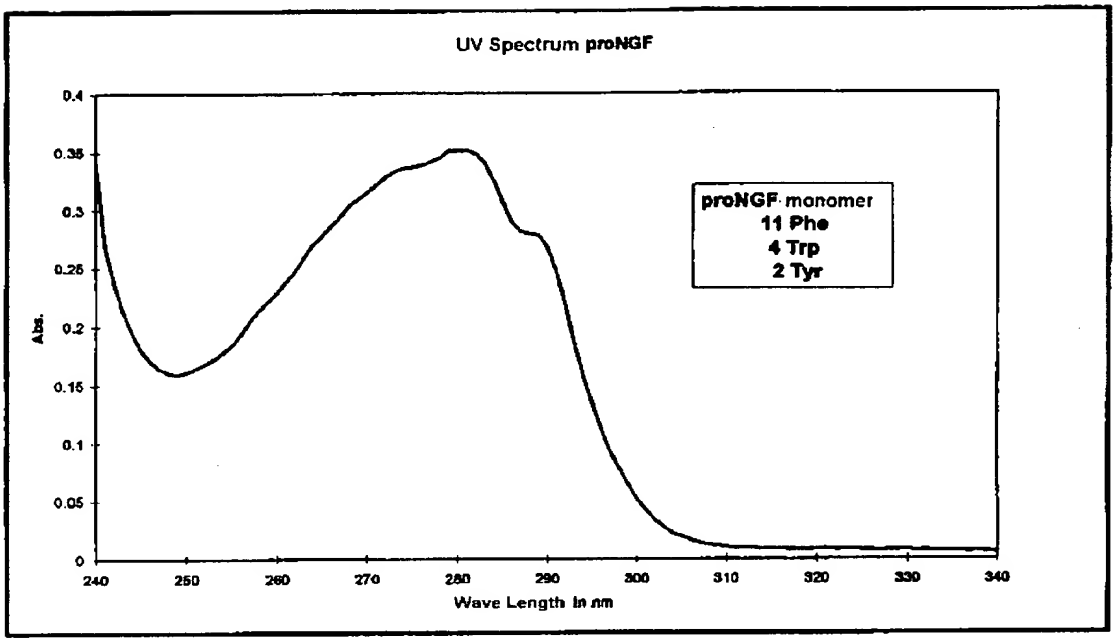

Fig. 6

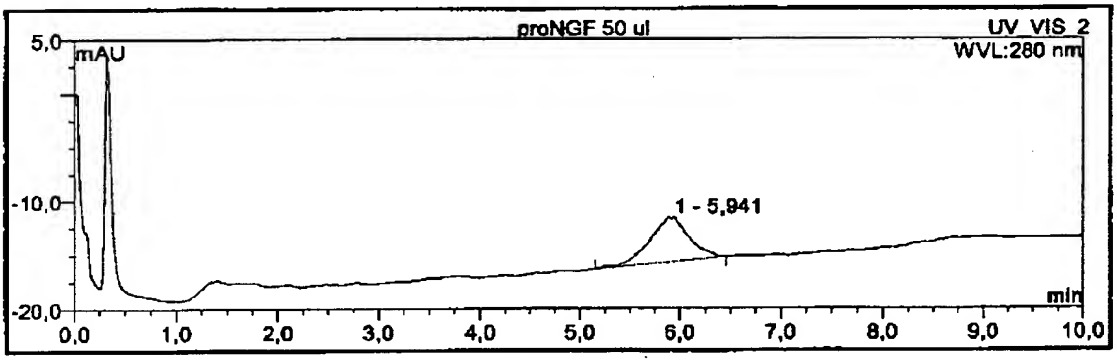

Fig. 7

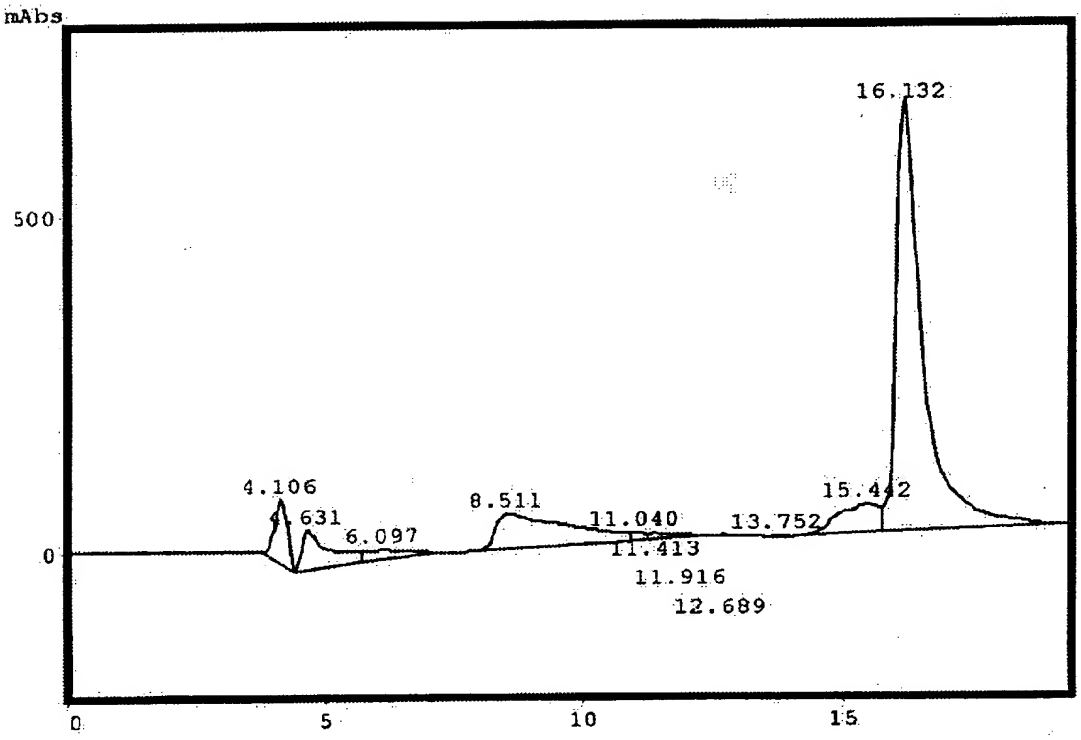

Fig. 8